PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, 15/87 — A1

(11) International Publication Number: WO 94/28148

(43) International Publication Date: 8 December 1994 (08.12.94)

(21) International Application Number: PCT/GB93/01162

(22) International Filing Date: 1 June 1993 (01.06.93)

(71) Applicant: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors: BAGNALL, Susan, Valerie; 222 South Hazel Avenue, Ames, IA 50010 (US). DRAYTON, Paul, Richard; 42 Notton Way, Lower Earley, Reading, Berkshire RG6 4AJ (GB). DUNWELL, James, Martin; 3 Hop Gardens, Henley-on-Thames, Berkshire RG9 2EH (GB). FRAME, Bronwyn, Ruth; 2117 Northwestern Avenue, Ames, IA 50010 (US). SPARKS, Caroline, Ann; 4a Half Moon Street, Bagshot, Surrey GU19 5AL (GB). THOMPSON, John, Alexander; 551 Reading Road, Winnersh, Wokingham, Berkshire RG11 5HJ (GB). WANG, Kan; 1623 Northwestern Avenue, Ames, IA 50010 (US). WILSON, Herbert, Martin; 1915 Stevenson Drive, Ames, IA 50010 (US).

(74) Agent: HUSKISSON, Frank, Mackie; Zeneca Limited, Group Patent Services Dept., P.O. Box 6, Bessemer Road, Welwyn Garden City, Herts AL7 1HD (GB).

(81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: GENETIC TRANSFORMATION METHOD

(57) Abstract

A method of introducing a nucleic acid into a plant cell comprises providing a culture of plant cells, placing proximate the plant cell culture a multiplicity of elongate needle-like bodies, such as silicon carbide whiskers and silicon nitride whiskers, and subjecting the said cell and bodies to physical motion so as to create relative movement, and thereby collisions, between the bodies and the cells whereby, on collision of a body and a cell, the cell is breached providing means for ingress of nucleic acid to the cell. The method is particularly applicable to cell culture types from which whole plants may be regenerated and its intended use is for the genetic transformation of cereal crops such as corn *Zea mays*.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

GENETIC TRANSFORMATION METHOD

This invention relates to the introduction of DNA into cells, particularly plant cells in order to effect transformation thereof by the inserted DNA.

In order that a cell may be efficiently transformed, certain requirements must be satisfied. First the gene to be inserted must be assembled within a construct which contains effective regulatory elements which will drive transcription of the gene. Next, there must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. The probability of integration may be improved by certain means but, in general, integration is simply a matter of uncontrolled chance. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

This invention is concerned with the second of these requirements, the transport of the gene construct through the cell wall. The first requirement is a matter for the skills of molecular engineering and the question of regenerability is a problem for the cell biologists and experts in tissue culture.

Plant cells are more difficult to transform than bacterial or animal cells because the presence of hard cell walls presents a barrier to insertion of the construct through that wall. However, many dicotyledonous plant species can be transformed using the natural gene transfer ability of Agrobacterium tumefaciens or Agrobacterium rhizogenes. Monocotyledonous plants, to which class the most agriculturally important cereals belong, remained for a long time recalcitrant to transformation on two counts. Firstly, such species are not

within the host range of Agrobacterium and secondly regenerable cell types have been difficult to find. However, recent work has given methodology for the creation and maintenance of regenerable maize callus and cell suspension cultures: see Armstrong C.L. and Green C.E.; Planta Vol. 164, pages 207-214 (1985) "Establishment and maintenance of friable embryogenic maize callus and the involvement of L-proline"; and, Kamo K.K. and Hodges T.K.; Plant Science Vol.45, pages 111-117 (1986) "Establishment and characterization of long-term embryogenic maize callus and cell suspension cultures"

In "Gene Transfer to Cereals: An Assessment"; Potrykus, Biotechnology 8, 535-542, June 1990, there is an assessment of all the then known methods of transforming cereals. The problems associated with the various methods are summarised in that paper.

Thus the choice of method for the transformation of plant cells tends to be limited to those which are convenient for the target plant type. As a generalisation, dicotyledonous plants are relatively easy to transform whereas monocotyledonous plants are very difficult, there being only a few techniques available in respect of which success has been reported, and that with very low success rate. There is, therefore, a need to make available new techniques for transformation of monocotyledonous plants.

One method which is claimed to transform cereal plant cells is the procedure known as "microinjection" where, under the microscope, a DNA construct is injected from a hollow needle into a target cell. A variant of that procedure is the rupturing of the cell wall with a needle, the DNA being added to the surrounding medium and allowed to diffuse into the cell through the break in the cell wall. This variant is known as "micropricking". Both of these procedures require a high degree of manipulative skill by the operator and are very time consuming.

It is probably true to say that to date the most effective methodology for the introduction of gene constructs into monocotyledonous cells has been the so-called "biolistics" method in which high density metallic particles, usually of tungsten or gold, are coated with the gene construct and are propelled by an explosive release of gas at a target cell culture. This alternative approach abandons the high precision of targeting which is inherent in microinjection and micropricking, in favour of a rapid "pepperpot" approach which enables large numbers of cells to be "hit" in a short time, giving a large number of putative transformants for screening.

Effective though the biolistic method may be, it requires expensive hardware and, although rapid by comparison with some other methods which have been attempted, is time-consuming. It does, however, achieve high numbers of insertions at each bombardment. One problem with this technique is the effect of the blast of expanding gas on the target tissue. Another is the difficulty of aiming the projectile shower at a selected area of the target.

Mixing of plant cells with plasmid DNA a sub-micron diameter fibres or whiskers is a simple and inexpensive alternative transformation method There have been two published reports of corn transform- ation using silicon carbide whiskers. The first described transient GUS expression in Black Mexican Sweet (BMS) corn suspension cells (Kaeppler et al., 1990). The same group have recently published their results on stable transformation of BMS and tobacco (Kaeppler et al., 1992). In the corn system a mean of 3.4 BASTA-resistant BMS colonies were recovered from each vortex-treated sample of cells (300µl packed cell volume) using a BAR and GUS-containing plasmid. Sixty five per cent of these herbicide-resistant colonies expressed GUS. (Kaeppler H.F., Gu W., Somers D.A., Rines H.W, Cockburn A.F. (1990) "Silicon carbide fiber-mediated DNA delivery into plant cells", Plant Cell Reports 9: 415-418,

and, Kaeppler H.F., Somers D.A., Rines H.W., Cockburn A.F. (1992), "Silicon carbide fiber-mediated stable transformation of plant cells", Theor. Appl. Genet. 84: 560-566

An object of the present invention is to provide an efficient and rapid method for the introduction of nucleic acids into plant cells.

According to the present invention there is provided a method of introducing a nucleic acid into monocotyledonous plant cells, comprising providing a culture of plant cells, placing proximate the plant cell culture a multiplicity of elongate needle-like bodies and subjecting the said cell and bodies to physical motion so as to create relative movement, and thereby collisions, between the bodies and the cells whereby, on collision of a body and a cell, the cell wall is breached providing means for ingress of nucleic acid to the cell.

Preferably the needle-like bodies are selected from the group consisting of silicon carbide whiskers and silicon nitride whiskers.

It may be preferred to pre-mix the needle-like bodies and nucleic acid and thereafter add the pre-mix to the cell culture.

When whole plants are required it is preferred that the said cell culture is of plant cells capable of regeneration into whole plants.

The invention is particularly applicable to a suspension culture of regenerable cells of Zea mays.

The physical motion applied to the cells, bodies and nucleic acid may be rotational, reciprocating or oscillating motion.

Preferably the nucleic acid is a DNA capable of genetically transforming the said cell.

In practice, the needle-like bodies and DNA are mixed and then added to the cells and the mixture agitated. However, it

makes little difference the order of addition and agitation is used. One embodiment of the invention comprises mixing the DNA and needle-like bodies (herafter referred to simply as "fibres) then adding this mixture to the cell suspension. The final mixture is vortexed together. The cells can then be incubated, and tested for expression of recombinant DNA. It has been found that the efficiency of DNA delivery varies according to the conditions; it is affected by several factors including the following: vortex time; cell suspension type (variation also found by H F Kaeppler et al, 1990, Plant Cell Reports, 9, 415-418); cell suspension age; osmolarity of culture medium; type of fibres; number of fibres present; type of DNA construct; concentration of DNA. Other factors which may affect DNA delivery include: the physical mixing methods used; the size, shape and uniformity of the fibres; the topology of the DNA (eg. linear, supercoiled); the presence of "carrier" DNA alongside the transforming DNA.

Many fine fibrous materials are known which may be used as the needle-like bodies in this invention, for example alumina, silica, titania, zirconia, boron, carbon, compounds such as the carbides, and glass fibre, but some of these materials have been implicated in causing cell lysis and death of animal cells but this would not necessarily preclude their use in this invention as it is known that they may be coated with a biocompatible coating to prevent such disadvantageous effects. Indeed the provision of such a coating may be advantageous in that it may provide a surface with which transforming DNA may be more conveniently bound, thus improving delivery of the DNA to the interior of the cell. Suitable such coatings may be synthetic resinous materials, surfactants and benign soluble materials such as alginate or gelatin.

Silicon carbide (SiC) is a ceramic compound used industrially in the manufacture of cutting tools and to reinforce composite materials. Silicon carbide can be produced

in the form of single crystals which are usually referred to as "whiskers" rather than fibres. These terms are used interchangeably herein.

SiC whiskers of less than 1μm diameter are ideally suited for use in this invention because of their high strength and good oxidation resistance. In recent years man-made mineral fibres have been increasingly exploited as substitutes for naturally occurring fibres such as asbestos. This has been driven predominantly by the serious health risks associated with the mining and use of crystalline minerals of the asbestos family. Increasingly it is being recognised that these health hazards are related, in part, to the morphology of the materials being used.

In practice plant cell transformation results from the mixing of intact cells or tissues with silicon carbide whiskers and plasmid DNA. We believe the mechanism of penetration to be largely a physical process and that the mixing of whiskers and cells must result in breach of the plant cell wall by the whiskers. Under the electron microscope many cells can be seen to have been impaled by the whiskers. We have shown that whisker-mediated DNA transfer does not occur (i) with whiskers and cells in the absence of mixing or (ii) with cells simply mixed with DNA in the absence of whiskers.

The method of the invention is inexpensive, requiring no sophisticated equipment and few consumables, and a large number of samples can be treated quickly. Simple safety precautions ensure that there is no risk of exposure to air-borne whiskers.

In fundamental principle, this type of transformation utilises a procedure which penetrates the cell wall in a non-lethal manner. Such methods, then, seek to wound but not kill the cells. In investigating possible procedures, it may be assumed that if particular method is capable of killing the cells then by making the treatment less severe the method may be adapted to wound.

The invention will now be described, by way of illustration, in the following Examples.

Abbreviations used in the Examples:

BMS Black Mexican Sweet corn

GUS βglucuronidase

cfu(s) colour forming unit(s), in histochemical GUS assays

PCR polymerase chain reaction

2,4-D 2,4-dichlorophenoxyacetic acid

NAA naphthaleneacetic acid

PCV packed cell volume

AxB A188xB73 hybrid

Brief Description of the Figures

Figure 1 shows a comparison of two types of silicon carbide whiskers;

Figure 2 illustrates the difference in expression obtained with different types of materials;

Figure 3 illustrates the effect of using different mixers on transformation rate;

Figure 4 is a time-course for transformed BMS using p35S-PAC, silicon carbide whiskers and a Vortex Genie mixer;

Figure 5 shows the effect of mixing time on cell viability;

Figure 6 compares transformation rate of BMS with different types of mixers;

Figure 7 shows the effect of mixing time on transient GUS expression in BMS;

Figure 8 illustrates the effect on transient expression of pMRP8 in silicon carbide whisker treated corn line A5D1-C6;

Figure 9 shows a Southern blot of five bialaphos resistant GUS expressing callus lines:

LANES

1 Lambda EcoRI - HindIII (molecular weight marker)

2 Genomic HindIII 1

3 Genomic HindIII 2

4 Genomic HindIII 3

5 Genomic HindIII 4

6 Genomic HindIII 5

7 Genomic HindIII negative

8 Undigested genomic DNA (very faint fogging visible on the photographic film).

9 Undigested genomic DNA (very faint fogging visible on the photographic film).

10 Uncut plasmid DNA (pBAR-GUS)

11 Lambda PstI (molecular weight marker); and,

Figure 10 gives maps of all the plasmids used in the Examples.

The major physical and biological parameters found to influence silicon carbide whisker-mediated transformation of maize cell are described in the Examples. Results are given for transient expression and for stable (integrative) transformation.

The rapidity with which transient expression data can be accumulated makes this a useful tool for the optimisation of the variable parameters of the method. Using GUS as a reporter gene in histochemical assays enables estimation of the number of cells receiving and expressing DNA through the counting of blue colour forming units (cfu's). We have used transient expression (of GUS) as a means of confirming DNA delivery with the whisker transformation approach.

Transient gene expression in its own right can be of value in confirming the functionality of a construct or in performing quantitative construct comparisons and this is the case with whisker-mediated transformation.

Transient assays can assist the rapid identification of treatment parameters which bring about an increase in transformation frequency. However, under some circumstances, enhanced transient expression may not translate into an increase in stable transformation efficiency. This is

particularly the case where cell survival is compromised. In the Examples we have attempted to monitor cell survival where appropriate.

EXAMPLE 1

WHISKER TYPE INFLUENCES THE EFFICIENCY OF DNA DELIVERY

Whiskers of <1µm diameter were assessed with this transformation method. Silicon carbide whiskers from two sources have been compared. Those used in our initial evaluations were obtained from Goodfellow, Cambridge (dimensions 0.5 x 30 µm). The whiskers used were acquired from Advanced Composite Materials Corporation, Greer, South Carolina (Silar SC-9 whiskers, size range 0.6 x 10-80 µm).

GUS expression from p35S-PAC was measured histochemically after 2 days for BMS cells treated with these different whisker types using the Gallenkamp Spinmix vortex mixer. The results are shown in Figure 1. A two-fold enhancement was observed in terms of blue colour-forming units for the Silar whiskers.

Scanning EM micrographs showed that the Silar whiskers appeared more uniform than the Goodfellow whiskers with less clumping and debris apparent. These differences may explain the observed increase in expression; there may have been more whiskers in the Silar suspension capable of transforming cells, because of their uniformity. It is also possible that the Silar whiskers may contain less debris or 'toxic' by-products of manufacture.

In another experiment silicon nitride (0.7 x 2-25 µm) whiskers were compared with Silar whiskers. The results of this comparison, which also included 44µm glass beads and carborundum are shown in Figure 2.

From these results it is evident that only particles with a needle-like morphology deliver DNA effectively under these conditions, silicon nitride may be less effective than SiC in this comparison because of the larger whisker diameter.

The use of different whisker types which may benefit from

further evaluation. There may be alternative sources of silicon carbide or other whiskers/ fibres with more suitable dimensions that would deliver DNA to cells more efficiently than the types evaluated so far.

EXAMPLE 2

MIXING METHOD AND DURATION INFLUENCES TRANSIENT EXPRESSION

Various mixing methods have been investigated in order to increase the level of transient GUS expression whilst monitoring the effect of these treatments on cell viability.

(i) BMS cells

Two standard laboratory vortex mixer models (the Gallenkamp Spinmix and Vortex Genie 2) have been directly compared. BMS cells, Silar SC-9 whiskers and p35S-PAC plasmid DNA were mixed for 60 seconds using either the Spinmix or Vortex Genie. Transient expression was monitored by histochemical GUS assay 2 days after treatment. The results are shown in Figure 3.

A two to four-fold increase in GUS expression was found using the Vortex Genie over the levels seen with the Gallenkamp Spinmix. A mean of 700 cfu's were obtained with the Vortex Genie in this experiment, Kaeppler et al. (1990) obtained 140 cfu's with BMS under similar conditions. The effect of increased vortex duration on transient expression and BMS cell survival was therefore investigated using the Vortex Genie. A five-fold increase in cfu's was observed between one and ten minutes of vortex treatment (Figure 4).

The largest increase in the number of GUS expressing cells came after three minutes of vortexing, with more than twice the number of cfu's seen after 60 seconds treatment. Observation of cells treated for 10 minutes showed that during vortexing the cell clusters became dissociated into small aggregates and single cells. Cell viability after vortexing was therefore determined to see if increasing vortex time led to cell death (Figure 5).

An initial drop in viability of about 20% was observed, with a further reduction of approximately 5% between the one and ten minute vortex times.

The effect of extended vortex duration (180 and 600 seconds) on the recovery of stably transformed callus has also been investigated (see below).

BMS cell/whisker agitation using an oscillating dental amalgam mixers (Mixomat and Silomat) has been evaluated. The results in Figure 6 show that when vortex time with the Spinmix was extended from 35 to 70 seconds the number of GUS cfu's increased. However, with the amalgam mixers the longer treatment time reduced GUS expression. Observation of the cells showed that after 60 seconds of Mixomat treatment there was significant cell dissociation and death. A time course of Mixomat treatments from 1 to 30 seconds was performed with BMS, the results of which are shown in Figure 7. GUS cfu's increase linearly up to 5 seconds of treatment but then reduce dramatically beyond 10 seconds, presumably as a result of reduced cell viability. To assess if the increased transient expression seen after 5 seconds of Mixomat treatment translated into more stable transformation events an experiment to compare 1 versus 5 seconds of treatment was performed.

(ii) AxB suspension cultures

An investigation into alternative mixing methods was undertaken comparing the Vortex Genie with the Mixomat amalgam mixer on the embryogenic suspension lines A5D1-C5 and A5D1-C6. It is apparent from Table 1 that, as with BMS, the Mixomat gave a significantly higher frequency of transiently expressing cells than the vortex mixer.

TABLE 1

Effect of mixing method on transient expression of p35S-PAC (cfu's)in suspension lines A5D1-C5 and C6

Mixing method	Vortex Genie		Mixomat	
Cell Line	C5	C6	C5	C6
Rep 1	112	176	969	1895
Rep 2	145	239	832	1049

Mixing times used were 3 minutes for the Vortex Genie and 5 seconds for the Mixomat.

To determine the optimum period for Mixomat treatment, a time course experiment was performed.

Table 2 illustrates the results, demonstrating that a treatment time of 5 seconds provided the maximum number of transiently expressing cells for cell lines C5 and C6.

TABLE 2

Effect of Mixomat time course on transient expression of p35S PAC in suspension lines A5D1-C5 and C6

Agitation Time (seconds)	C5	C6
1	387	940
5	792	2204
10	540	897
20	207	775
30	-	301

No osmotic pre-treatment was used and additional medium was excluded.

In order to evaluate the effect of Mixomat treatment on cell survival, C5 and C6 suspension cells were subjected to the same Mixomat time course with cell viability measured after 24 hours.

Up to 50% loss of viability occurred as a result of Mixomat treatment for 5 seconds. After one second of mixing (the minimum time setting on the machine), cell mortality was less than 25% and levels of transient expression (as shown in Table 2) were still reasonable, hence this period of treatment was used as standard in subsequent transformation experiments involving the Mixomat.

EXAMPLE 3

OSMOTIC TREATMENT OF CELLS

(i) AxB suspension cultures

Since the application of osmotic stress to target cells prior to particle bombardment (in the Biolistics method) is known to result in increased frequencies of both transiently expressing cells and stable transformants, an investigation into the utility of osmotic treatments prior to, and/or following whisker-mediated transformation of A5D1-C6 was undertaken.

The osmotic medium used was identical to that used before particle bombardment (H9CP+ with 45.4 g/l mannitol and 45.4 g/l sorbitol). Treatment consisted of immersion of 250 mg cells per replicate in 1 ml osmotic medium for 30 minutes. Controls were immersed in unamended H9CP+ for the same period.

Results given in Table 3 clearly demonstrate that osmotic pretreatment is beneficial with respect to transient transformation. It would appear however that the treatments following whisker mixing offered no advantage.

TABLE 3

Influence of osmotic treatment on transient expression of GUS from p35S-PAC in suspension line A5D1-C6

Replicate	Control (cfu's)	Pretreatment (cfu's)	Pre + Post treatment (cfu's)	Post-treatment (cfu's)
1	459	1184	1023	255
2	396	1302	1395	445
3	565	1489	1341	436

Mixing treatment was 1 second with the Mixomat

Various modifications to this method have also been assessed. These include reduction in the duration of osmotic treatment and treatment in a flask with shaking rather than in the Eppendorf tube. Results are presented in Table 4 for some of these experiments:

TABLE 4

Effect of various osmotic treatments on transient GUS expression in regenerable culture

		Pre-treatment				
		In Eppendorf		In Shaking Flask		
Expt.	Cell Line	10 min	30 min	0 min	10 min	30 min
1	C6	928	973	626	-	-
2	C6	-	768	296	-	1284
3	C12	-	-	270	955	1187

These results show the beneficial effect of performing osmotic treatment in a shaking flask. It may be that this improvement is a consequence of increased cell aeration and

survival. A further benefit of flask osmotic treatment is improved experimental (time/effort) efficiency.

(ii) Effect of Osmotic Treatments on BMS cultures

Surprisingly, osmotic pre-treatment of BMS almost totally eliminated DNA delivery as judged by transient GUS expression (maximum of 25 cfu's). Microscopic observation of cells immediately after treatment suggested that this was the result of excessive cell plasmolysis. Since these same conditions work well with regenerable lines, it is apparent that BMS responds differently with respect to osmotic conditioning, presumably because of differences in cell size and degree of vacuolation.

EXAMPLE 4

AGE OF CELL SUSPENSION

The influence of suspension age (relative to subculture) on competence for transformation has been investigated for A5D1-C8 and C12. Results are shown in Table 5.

TABLE 5

Transient expression in A5D1 lines at different stages relative to subculture

	Day after subculture (cfu's) n=3				
	Day 0	Day 1	Day 2	Day 3	Day 4
C8	638±84	938±51	448±27	333±40	398±28
C12	357±79	590±104	363±25	244±14	248±23

Cells were osmotically pre-treated for 30 minutes on the shaker. Mixing was performed with the Vortex Genie for 60 seconds.

Analysis of variance on these results indicated a significant day effect for both cell lines.

Mean transient counts at day 1 were significantly higher than the other suspension ages tested. Days 3 and 4 did not differ significantly from each other. Day 0 cfu's were

significantly higher than day 4 for C8 but not for C12. It may be of value to identify the optimum suspension age for each new cell line treated, assuming that there is a direct, positive correlation between cfu's and stable clone production for this cell culture characteristic.

For BMS, optimum suspension age was found to be 2 days, during exponential growth of the culture.

EXAMPLE 5

PRE-MIXING OF WHISKERS AND DNA

We have evaluated the timing of DNA/whisker interaction to see if this affects the efficiency of DNA delivery to A5D1-C6 suspension cells. Normally whiskers and DNA are vortex mixed for 60 seconds prior to addition of cells. Various pre-mixing periods were assessed and compared with no pre-mixing, the results are shown in Figure 8. Mixing involved 1 second of Mixomat treatment. The plasmid was pMRP8

From these results it is apparent that pre-mixing of whiskers with DNA is not necessary to achieve efficient DNA delivery to C6 cells. This does not preclude the possibility that a rapid spontaneous interaction occurs between whiskers and DNA based on their relative charges.

EXAMPLE 6

CONFORMATION OF PLASMID DNA

Linearised and supercoiled DNA have been compared in BMS. The results are shown in Table 6.

TABLE 6

Comparison of supercoiled versus linearised p35S-PAC in BMS cells

DNA form	Mean cfu's	Fluorimetric GUS nMol 4-Mu/mg/hour
Supercoiled	505	30.3
Linearised	301	4.7

Values are the mean of 3 replicates. Vortex Genie (60s) treatment was used.

It is clear from this result that supercoiled DNA is preferable to linearised DNA in terms of transient GUS expression. This is in broad agreement with published work on "direct gene transfer." The difference seen here in fluorometric GUS activity is greater however than we saw when the same comparison was performed in AxB suspension protoplasts.

EXAMPLE 7

ZYGOTIC EMBRYOS, EMBRYOGENIC CALLUS AND PROTOPLASTS

(i) Whisker treatment of immature maize zygotic embryos

Because of the ease of plant regeneration from responsive genotypes, zygotic embryos are an attractive target tissue in corn for transformation. An investigation into the utility of the whisker method for DNA transfer to embryos was undertaken.

Initial studies used the fluorometric GUS assay to examine the outcome of enzyme pretreatment and vortex time course on whisker treatment of 11 day old A188 embryos. Neither degrading the cell walls nor increasing the vortex time resulted in increased transient expression, although samples showing up to 23 times background GUS activity were identified.

Comparison of Mixomat treatment for 1 or 3 seconds with vortexing for 5 minutes (using 11 day old FH24 embryos) identified vortexing without medium present to be the least damaging treatment and to give the highest frequency of cfu's per embryo in histochemical assays. Whilst up to 85% of embryos were found to have blue spots, the mean was only 3 per embryo (range 1-12). It is assumed that only cells on the surface of the embryo will receive DNA using whiskers.

Evaluation of osmotic pretreatment for 30 or 60 minutes with 11 day old UE95 embryos identified a trend of decreasing transient expression with increasing pretreatment period (55%

and 37% respectively of embryos had cfu's).

(ii) Whisker treatment of type III callus lines

"Type III" Be70 and Be81 callus lines were used in transformation assessments with whiskers using the Mixomat. To ensure that both lines received the optimum period of Mixomat treatment, a time course experiment was performed. No osmotic pretreatment was used in this experiment, the results of which are presented in Table 7.

TABLE 7

Mixomat time course applied to Be70 and Be81 type III callus lines

Agitation Time (seconds)	Be70 GUS cfu's	Be81 GUS cfu's
1	28	21
5	63	76
10	54	34
20	40	11
30	25	16

From the time points investigated, the 5 second treatment resulted in maximum transient expression for both lines.

Subjecting the type III tissue to 30 minutes osmotic pretreatment prior to transformation (Mixomat 1 second) resulted in a 6 (Be70) and 8 (Be81) fold increase in transient expression over controls (Table 8).

TABLE 8

Effect of osmotic pretreatment of type III lines

Line	Control cfu's	Pretreatment cfu's
Be70	19 18 24	144 168 133
Be81	38 25 22	282 130 297

(iii) Whisker treatment of protoplasts

We were interested to learn whether whiskers could deliver DNA to corn protoplasts. An experiment was performed with PEG uptake as a control using the conditions shown in Table 9.

TABLE 9

GUS expression from BxA protoplasts with whiskers and PEG uptake

Treatment Number	DNA	Whiskers	Vortex	GUS Expression nMol/mg/hour
1	+	+	+(1)	385
2	+	+	+(2)	520
3	+	PEG	-	7618
4	+	-	-	0
5	+	-	+	0
6	-	+	+	0

The Vortex Genie II was used at two speed settings:

1 setting= shaker 3

2 setting= vortex 1

Values are the mean of three replicates

These results show that whisker treatment of protoplasts results in DNA delivery albeit at a lower rate than that achieved with PEG treatment. This would suggest that cell/protoplast penetration by whiskers occurs rather than abrasion which would result in loss of protoplast viability to the exclusion of GUS expression.

EXAMPLE 8

VERIFICATION OF STABLE TRANSFORMATION IN BMS

The first experiment involved a repeat of the conditions described by Kaeppler et al. (1992) with the exception that the Gallenkamp Spinmix was used and selection was performed using medium containing Bialaphos rather than BASTA. Selection with 1 mg/l bialaphos was applied 48 hours after treatment, after 7 days at this level it was increased to 5 mg/l.

A mean of 32 bialaphos-resistant colonies per treatment were recovered in this experiment. Seventy per cent of these colonies expressed GUS and were PCR positive. The remaining colonies (19 in total) did not express GUS. PCR established that 14 of the 19 colonies contained the GUS gene ie. they were transformed but non-expressers. The remaining 5 GUS-negative colonies were also PCR negative ie. they were "escapes".

In summary, 92.0% of all the colonies contain the GUS gene in some form and these colonies were stably transformed (ie. 29 stably transformed colonies were obtained per treatment).

Five of the GUS expressing PCR-positive lines were used in Southern analysis.

The resulting blot (Figure 9) showed that the BAR gene was present in the nuclear genome of all five of the colonies which were used in Southern hybridisation. Rearrangement of the BAR gene also appears to have taken place in some of the colonies.

The number of stably transformed lines per treatment obtained in this experiment (29) is clearly higher than that obtained by Kaeppler et al. (1992). This could be due to the

use of bialaphos rather than BASTA selection or may be attributed to the different BMS cell line used. A transient expression experiment performed at the same time as this stable transformation study gave an average of 100 cfu's per treatment, indicating a transient to stable transformation ratio of nearly 30% compared with the 10% obtained by Kaeppler et al.

EXAMPLE 9

EFFICIENCY OF STABLE TRANSFORMATION IS INFLUENCED BY SELECTION CONDITIONS

The choice of level and timing of selective agent application is a key consideration in the development of any transformation system. It is important to ensure that the balance between selection stringency and the growth of non transformed tissue is maintained without reducing the growth of transformed callus.

A second stable transformation experiment was performed with BMS to evaluate the effect of initial bialaphos selection level on the number of callus lines subsequently recovered.

Three levels of bialaphos selection were compared; 1, 2 and 5 mg/l. Here, selection commenced one day after treatment with whiskers and continued for 7 days, after which the selection level for all treatments was maintained at 5 mg/l bialaphos.

A total of 517 bialaphos-resistant colonies were recovered from 16 plates (16 treatments) in this experiment (Table 10). Approximately 90% of all the colonies recovered from the plates were GUS expressing, irrespective of the level of bialaphos selection applied.

From these results it can be seen that the 1mg/l initial bialaphos selection produced the highest number of transformed callus lines (43 per treatment). This concentration of bialaphos has since been adopted in our standard BMS/whisker transformation protocol.

TABLE 10

Effect of bialaphos selection level on recovery of stably transformed BMS callus

Initial Level of Bialaphos selection (mg/litre)	1	2	3
Number of Treatments	7	7	2
Number of callus lines selected	304	173	39
Callus lines	43	25	19
Histochemical GUS assay - positive	278(91%)	157(91%)	33(85%)
Histochemical GUS assay -negative	26(9%)	16(9%)	6(15%)

The Gallenkamp Spinmix was used to mix cells, whiskers and DNA for 60 seconds

Selection was initiated at the levels shown one day after whisker treatment

Selection was increased to 5mg/l after seven days at this level

EXAMPLE 10

EFFECT OF MIXING DURATION AND METHOD ON STABLE BMS TRANSFORMATION

(i) Vortex duration and vortex model

An experiment to evaluate stable transformation of BMS following 60,180 and 600 seconds of vortex treatment (using the Vortex Genie II) was performed. This was prompted by transient expression results which showed a two-fold increase in cfu's when vortex duration was increased from 60 to 180 seconds and a five-fold increase from 60 to 600 seconds.

After 60 seconds of Vortex Genie mixing a mean of 73

bialaphos-resistant callus lines were obtained per treatment using the selection scheme arrived at above. This compares favourably with the number of calli obtained using the Gallenkamp Spinmix (mean 43 lines per treatment).

Increasing the vortex period to 180 seconds did not increase the number of callus lines obtained (75 per treatment developed). The increase in transient expression does not therefore translate into an increase in stable transformation rate in this case, presumably as a result of reduced cell survival after the longer vortex period.

Following 600 seconds of mixing, no stably transformed lines were recovered and the re-growth of callus on unselected plates was very poor compared with controls.

These results highlight the importance of considering cell survival when attempting to equate transient expression with stable transformation for a given treatment.

(ii) Mixomat treatment

An experiment to evaluate stable BMS transformation after Mixomat (amalgam mixer) treatment was also performed. One second of mixing was used and the effect of culture medium inclusion/exclusion was assessed. Transient expression results suggested that the exclusion of additional culture medium resulted in an increase in cfu's, presumably because of an increase in the number or force of cell-whisker collisions.

A mean of 65 bialaphos-resistant callus lines were recovered from each treatment after 1 second of Mixomat treatment with medium present. This figure increased to 160 callus lines per treatment when medium was excluded, confirming the earlier transient expression result. This represents the highest number of stably transformed BMS callus lines obtained per treatment with whisker transformation to date.

After 5 seconds of Mixomat treatment there was a dramatic reduction in the number of stably transformed lines obtained (2 per treatment in the absence of medium). Transient expression

for 5 seconds of treatment was around three-fold higher than that for 1 second of treatment.

There is probably scope for optimisation of stable transformation rates through the exploration of alternative mixing methods and periods. However our results clearly indicate that transient expression alone does not always enable improved conditions to be identified. Careful consideration of the effect of mixing conditions on cell survival for each culture line must be made.

EXAMPLE 11

STABLE TRANSFORMATION OF REGENERABLE CULTURES

A series of experiments has been carried out with regenerable suspension cultures. A total of nine callus lines resistant to 1 mg/l bialaphos have developed and plants have been regenerated from 6 of these. The experiments which have resulted in callus production are summarised in Table 11.

TABLE 11

Summary of bialaphos-resistant clones obtained from regenerable suspensions

Cell Line	Mixing	Callus Analysis		Plants	Clone Name
		GUS assay	PCR(GUS)		
C8	MM	+	+	+	UC2
C8	MM	-	+	+	RU2
C8	VG	+	+	+	RR2
C6	VG	+	+	+	UB2
C8	VG	+	nd	+	UJ2
C12	VG	+	nd	+	UA2
C12	VG	-	nd	+	UI2
C8*	VG	+	nd	+	UZ15
C8	MM	+	nd	+	WB2
C12**	VG	-	nd		
C13	VG	+	nd		

Mixing treatments: MM; Mixomat (1 second), VG; Vortex Genie (60 seconds)

nd; not determined

Named clones have produced plants currently in the glasshouse

The transforming plasmid was pBAR-GUS except for * which was from pMRP7 and ** which was from pMRP8.

Various osmotic treatments were assessed in these experiments.

Transient expression was monitored in most of the experiments summarised in Table 11, however a clear

relationship between treatment, transient expression and clone production was not apparent in these experiments.

EXAMPLE 12

ANALYSIS OF BIALAPHOS-RESISTANT CALLUS

Table 10 summarises the results of GUS and PCR assays carried out on the 9 clones obtained to date. Most of the clones are GUS expressing and 4/4 of those assayed by PCR (for GUS) were positive.

EXAMPLE 13

REGENERATION AND ANALYSIS OF TRANSGENIC PLANTS

Using the protocol described below in respect of regeneration from bialaphos-resistant colonies, plants have been regenerated from six of the nine clones obtained (indicated in Table 10). Plants from five of these six are currently in the glasshouse. There are 18 plants from clone RR2 and 20 plants from clone RU2.

Analysis:

(i) RR2

Two plants have been BASTA "leaf paint" assayed; both were positive.

Ten plants have been GUS assayed (histochemical assay on leaf samples); all were positive.

One plant has been PCR assayed (for GUS) and was positive.

(ii) RU2

One plant has been leaf paint assayed and was positive.

Eight plants have been GUS assayed; all were negative (the callus was also non-expressing).

MATERIALS AND METHODS

The materials and methods used in the Examples are:

CELL CULTURES

Black Mexican Sweet corn (BMS) culture

A finely dividing, non-regenerable cell suspension of Black Mexican Sweet corn (BMS) was used. This line has been

maintained in our laboratories for over 5 years.

Subculturing of BMS is performed every 7 days by transferring 10 ml of culture into 80 ml fresh BMS medium, the cultures are grown at 25°C on a rotary platform shaker (120 rpm).

Experiments were carried out on 2 day old cell suspensions. A packed cell volume of 250 µl was used for each treatment. This was achieved by centrifuging the contents of a flask of BMS at 600 rpm for 3 minutes, removing the supernatant and adding the same amount of fresh BMS medium to packed cell volume. 500 µl of this suspension was then used for each treatment (250 µl of cells and 250 µl of medium).

Embryogenic Corn suspension cultures

AxB suspensions derived from cryopreserved tissue (generic designation A5D1) were used for whisker treatment. The cultures are subcultured twice weekly by transferring cells and conditioned medium into fresh H9CP+ medium. Cells are growm at 28°C, 125 rpm.

Culture medium from a 1 day old culture was, where appropriate, replaced with fresh H9CP+ plus osmoticum (0.5M sorbitol/ mannitol) and returned to 125 rpm for 30 minutes.

AxB CELL SUSPENSION INITIATION AND CELL LINE DERIVATION

Zygotic embryos, 10-12 day old, from a greenhouse-grown A188xBe70 hybrid were plated on N6 base medium containing 6mM proline, 2% sucrose, 2mg/l 2,4-D and 0.3% Gelrite. Proliferating Type II structures from a single responding embryo (A5Di) were cultures on the same medium and the callus used to initiate a cell suspension. Approximately 3g of callus were added to 22ml liquid H9CP+ and 1ml of coconut water and the flask maintained at 28°C, 128rpm and subcultured every 3½ days.

DERIVATION OF CELL CULTURES USED IN WHISKER EXPERIMENTS

A5D1-C6: derived from a cryopreserved A5D1 suspension. Thawed callus was used to initiate the susupension two weeks

after thawing. At the time of whisker treatment the suspension was well established and consisted of small aggregates. The total time in suspension was around 12 months.

A5D1-C8: derived from a cryopreservation of A5D1-C6 suspension. The thawed callus was used to initiate the suspension A5D1-C8. The suspension was characterised by a dispersed but large-aggregate type. The total time in suspension was 10-11 months.

A5D1-C12: derived from cryopreserved A5D1 which had spent 8-10 months in suspension at the time of used in whisker transformation. It is a medium-sized aggregate suspension.

ZYGOTIC EMBRYOS

Corn inbred line FH24 was self-pollinated and embryos excised at 10-15 days after pollination. These embryos were used in whisker-mediated transformation.

Embryos at the approriate stage oif develoment may be cultured on a suitable medium and they will form an embryogenic callus from which plants may be directly regenerated. This may be achieved with the majority of corn genotypes via a TypeI callus (compact nodular tissue).

Certain genotypes will form Type II and Type III embryogenic callus from embryos either directly or from another callus type. Type II is composed of somatic embryos in various stages of maturation but early, that is, stalked embryos on basal callus is preferentially selected at subculture.

Type III callus is formed only rarely and is much more friable, that is, it is easily dispersed and does not have any distinct embryos on it. This is ideal tissue for transformation because it is accessible for DNA delivery and will readily form cell suspension cultures. It has to be selected visually and preferentially at subculture.

PROTOPLASTS

The protoplasts referred to herein were isolated from a B73xA188 embryo callus (Type III)- derived cell suspension

culture. The protoplasts were obtained by a standard procedure with enzymatic digestion of the suspension in 2% cellulase RS and 0.2% pectolyase Y-23.

PLASMID DNA

p35S-PAC was used for most of the transient expression experiments. Plasmid p35S-PAC contains the cauliflower mosaic virus (CaMV) 35S promoter and the intron 1 of maize alcohol dehydrogenase (Adh1) fused to the GUS (uid A) gene and the CaMV polyadenylation signal.

pBAR-GUS was used for most of the stable transformation experiments. This plasmid contains the BAR gene fused to the 35S CaMV promoter and the intron 1 of maize Adh1 joined in opposite orientation to the GUS gene which is under the control of the Adh1 promoter and intron 1 of maize Adh1

Figure 10 gives maps of all the plasmids used in these experiments.

PREPARATION OF SILICON CARBIDE WHISKERS

Dry whiskers were always handled in a fume cabinet, to prevent inhalation and possible lung damage. These whiskers may be carcinogenic as they have similar properties to asbestos. The Silar SC-9 whiskers were provided by the Advanced Composite Material Corporation Greer, South Carolina,USA. The sterile whisker suspensions were prepared in advance as follows. Approximately 50mg of whiskers were deposited into a pre weighed 1.5 ml Eppendorf tube, which was capped and reweighed to determine the weight of the whiskers. The cap of the tube was perforated with a syringe needle and covered with a double layer of aluminium foil. The tube was autoclaved (121°c, 15psi, for 20 minutes) and dried. Fresh whisker suspensions were prepared for each experiment, as it had been reported that the level of DNA transformation when using fresh suspensions was higher than that of older suspensions (Kaeppler et al.1992). A 5% (weight/volume) whisker suspension was prepared using sterile deionised water. This was vortexed for a fev seconds to

suspend the whiskers immediately before use.

DNA TRANSFORMATION INTO CELLS

All procedures were carried out in a laminar air flow cabinet under aseptic conditions. The DNA was transformed into the cells using the following approach. Specific modifications to this method are indicated in the text.

Cell and whisker suspensions were pipetted using cut down Gilson pipette tips. 100µl of fresh BMS medium (see appendix 1) was measured into a sterile Eppendorf tube. To this was added 40µl of the 5% (w/v) whisker suspension and 25µl (1mg/ml) of the plasmid DNA, which was vortexed at top speed for 60 seconds using a desktop vortex unit (Vortex Genie 2 Scientific industries, Inc). Immediately after this period of vortexing, 500µl of the cell suspension was added ie 250µl of packed cells (see 3.1 for preparation of cell suspension). The Eppendorf tube was then capped and vortexed at top speed for 60 seconds in an upright position. The same procedure was used to transform the other cell lines.

CELL VIABILITY

After the cells had been treated and incubated, 2ml of fluorescein diacetate (3 drops of 5mg/ml FDA to 10 ml of water) was added to each sample and left for 5 minutes. The total number of cells and the number of fluorescent cells were counted, using a haemocytometer slide viewed under ultra violet light. From this the percentage of living cells was determined.

GUS ASSAYS

For BMS, following vortexing, 500µl of BMS medium was added to each tube, the contents of which was poured into a 50 x 20mm disposable petri dish. A further 1 ml of BMS medium was pipetted into the tube, gently shaken and poured into the petri dish, to ensure that all the cells had been removed from the tube. The plates were sealed with Nescofilm and incubated in the dark for 48 hours at 25°c.

Fluorometric GUS assays were performed and results calculated according to the method produced by Caroline Sparks (available on request).

Histochemical assays were performed using 2 ml of the following assay buffer /sample:

50 ml 0.1 M dibasic/monobasic sodium phosphate
80 mg Potassium ferricyanide
105 mg Potassium ferrocyanide
0.06% Triton X-100
0.1% Dimethyl sulphoxide
500µl X-gluc (5% solution in DMF)

Tissue was incubated for 24 hours at 37°C.

SELECTION OF STABLE TRANSFORMANTS

BMS

Samples of BMS cells treated with pBAR-GUS and silicon carbide whiskers and control samples treated with whiskers alone, were dealt with as follows:

i) Immediately after vortexing the sample in the Eppendorf tube 500µl of fresh BMS medium was added.

ii) The contents of the tube were poured onto a 12.5 cm disc of Whatman no.2 filter paper, overlying 75 ml of BMS medium (0.6% Sea Plaque agarose), in a 140 x 25 mm petri dish.

iii) A further 1 ml of BMS medium was pipetted into the tube, shaken briefly and poured onto the plate, in order to remove all the cells from the tube.

iv) The plates were agitated to distribute the cells as a thin layer over the filter paper, sealed with Nescofilm and incubated in the dark at 25°C for 24 hours.

v) After this period of incubation the filter paper and the overlying cells were transferred onto the selection medium. This comprised 90 ml of BMS medium solidified with 0.6% Sea Plaque agarose, containing 1mg/litre of bialaphos in a 140 x 25 mm petri dish. The sealed dishes were incubated for 7 days at 25°C.

vi) After this period of selection on 1mg/litre of bialaphos the filter paper and the overlying cells were transferred onto selection medium containing 5mg/litre of bialaphos.

vii) The plates were sealed with Nescofilm and incubated in the dark at 25°C.

viii) The support filter papers and overlying cells were transferred onto fresh selection medium with 5mg/l bialaphos at two week intervals.

ix) After approximately 3 weeks of selection, putatively transformed cells growing on the selection medium were observed.

x) The resistant colonies were placed directly onto 2 ml of 2 mg/litre bialaphos selection medium (one colony per well in 25 well plates), once they had grown to approximately 3 mm in diameter. Subculturing was then carried out every two weeks onto fresh selection medium.

A5D1 suspension cultures

i) Treated cells were transferred to 4.7 cm diameter Whatman no.1 filters overlying 12 ml of N6 medium (2 mg/l 2,4-D) solidified with 3 g/l Gelrite in 5 cm dishes and cultured at 28°C in the dark.

ii) After 7 days the filters were transferred to fresh N6 medium containing 1 mg/l bialaphos.

iii) After a further 7 days the filters were transferred again to fresh N6 with 1 mg/l bialaphos.

iv) Tissue from one filter was dispersed in 5 ml of cool N61B medium with 3 g/l Sea Plaque agarose. The tissue/medium mixture was then spread onto the surface of two plates of N61B medium solidified with Gelrite.

v) Bialaphos-resistant callus was picked from embedded tissue 5-6 weeks later.

VERIFICATION OF STABLE TRANSFORMATION

Histochemical gus assay

A histochemical gus assay was performed on each individual

BMS colony once they had grown to approximately 5 mm in diameter on the 2 mg/litre bialaphos selection medium. The degree of staining of the gus positive colonies was observed. If colonies were gus negative for this assay it was possible that they were either escapes which could tolerate 5mg/litre of bialaphos, or that they were not expressing the GUS gene. In order to verify if these colonies were gus negative transformants or if they were "escapes", they were assayed with PCR.

Polymerase Chain Reaction (PCR)

PCR was used to amplify an internal region within the GUS gene consisting of 496 base pairs, using 2 oligonucleotides each consisting of a sequence of 27 bases.

PLANT REGENERATION FROM BIALAPHOS-RESISTANT CLONES

After picking from selection plates, callus was bulked on N61B medium. At subculture, type II embryogenic tissue from a stock plate was transferred to MS medium with 1 mg/l NAA and 6% sucrose (25°C, dark) for 2-3 weeks. Mature, opaque structures were moved to the light on MS medium with 0.25 mg/l NAA. Developing plantlets (ie. with both shoot and root visible) were transferred to 1/2 strength MS medium with 3% sucrose and no hormones in air exchange vials until the roots were well developed. They were then moved into hydroponics in the glasshouse, then into peat pots and finally into 3 gallon pots for growth to maturity.

CLAIMS

1. A method of introducing a nucleic acid into a plant cell, comprising providing a culture of plant cells, placing proximate the plant cell culture a multiplicity of elongate needle-like bodies and subjecting the said cell and bodies to physical motion so as to create relative movement, and thereby collisions, between the bodies and the cells whereby, on collision of a body and a cell, the cell wall is breached providing means for ingress of nucleic acid to the cell.

2. A method according to claim 1, in which the needle-like bodies are selected from the group consisting of silicon carbide whiskers and silicon nitride whiskers.

3. A method according to claim 1 or claim 2, in which the said bodies and said nucleic acid are pre-mixed and thereafter added to the cell culture.

4. A method according to claim 1 or 2 or 3, in which the physical motion applied to the cells, bodies and nucleic acid is rotational motion.

5. A method according to claim 1 or 2 or 3, in which the physical motion applied to the cells, bodies and nucleic acid is

reciprocating or oscillating motion.

6. A method according to any preceding claim, in which the nucleic acid is a DNA capable of genetically transforming the said cell.

7. A method as claimed in clain 6, wherein the said DNA is linear or supercoiled plasmid DNA.

8. A method as claimed in claim 6 or claim 7, in which the said DNA includes a selectable marker gene.

9. A method as claimed in claim 8, in which the said selectable marker gene specifies resistance to bialaphos.

10. A method according to any preceding claim, in which the said cell culture is of plant cells capable of regeneration into whole plants.

11. A method according to claim 10, in which the said cell culture is a suspension culture of regenerable cells of Zea mays.

12. A method as claimed in any of claims 1 to 9, in which the cell culture is of zygotic embryos.

13. A method as claimed in any of claim 1 to 9, in which the cell culture is of embryogenic callus.

14. A method as claimed in any of claim 1 to 9, in which the cell culture is of plant cell protoplasts.

15. A method as claimed in any preceding claim, in which the said cell culture is subjected to osmotic stress prior to contact with the needle-like bodies.

16. A method for the production of a transgenic monocotyledonous plant, comprising providing a culture of cells of the said plant of a type which is regenerable into whole plants, placing proximate the plant cell culture a multiplicity of elongate needle-like bodies and plasmid DNA containing a gene gene of interest and a selectable marker gene, subjecting the said cells and bodies to physical motion so as to create relative movement, and thereby collisions, between the bodies and the cells whereby, on collision of a body and a cell, the cell wall is breached providing means for ingress of nucleic acid to the cell, transferring the cells to a regeneration medium containing a selection agent appropriate for the marker gene and recovering surviving plantlets from the medium.

17. A method as claimed in claim 16, in which the said plant is Zea mays.

FIG. 1

COMPARISON OF SILLAR SC-9 FIBRES AND 0.5x 30um GOODFELLOW FIBRES (GALLENKAMP SPINMIX)

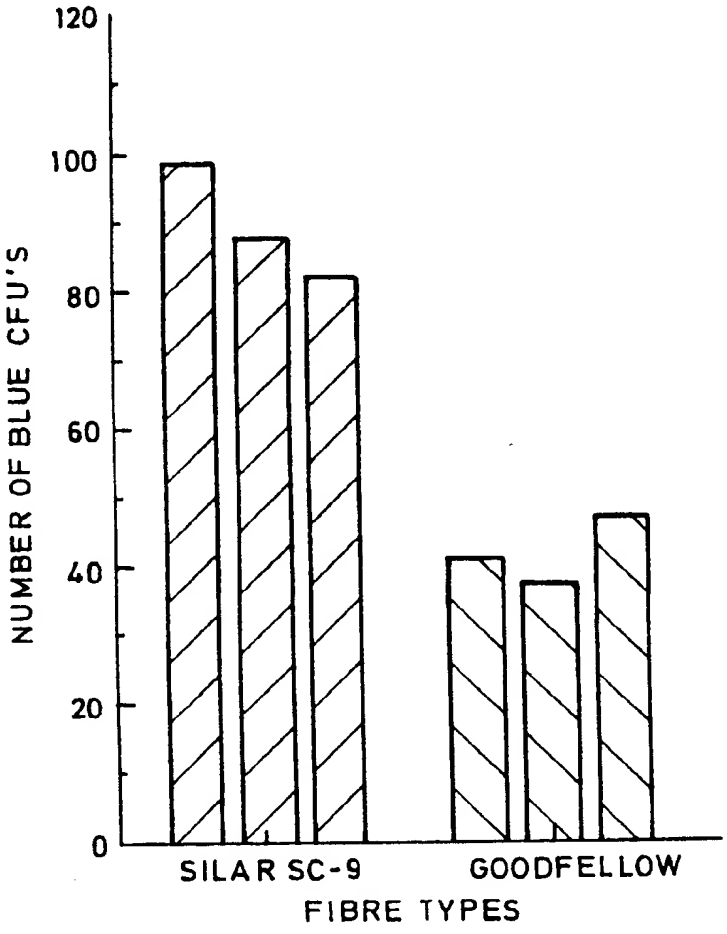


FIG. 2

COMPARISON OF MIXING MATERIALS ON p35S-PAC EXPRESSION IN ASD1-C6 SUSPENSION CELLS.

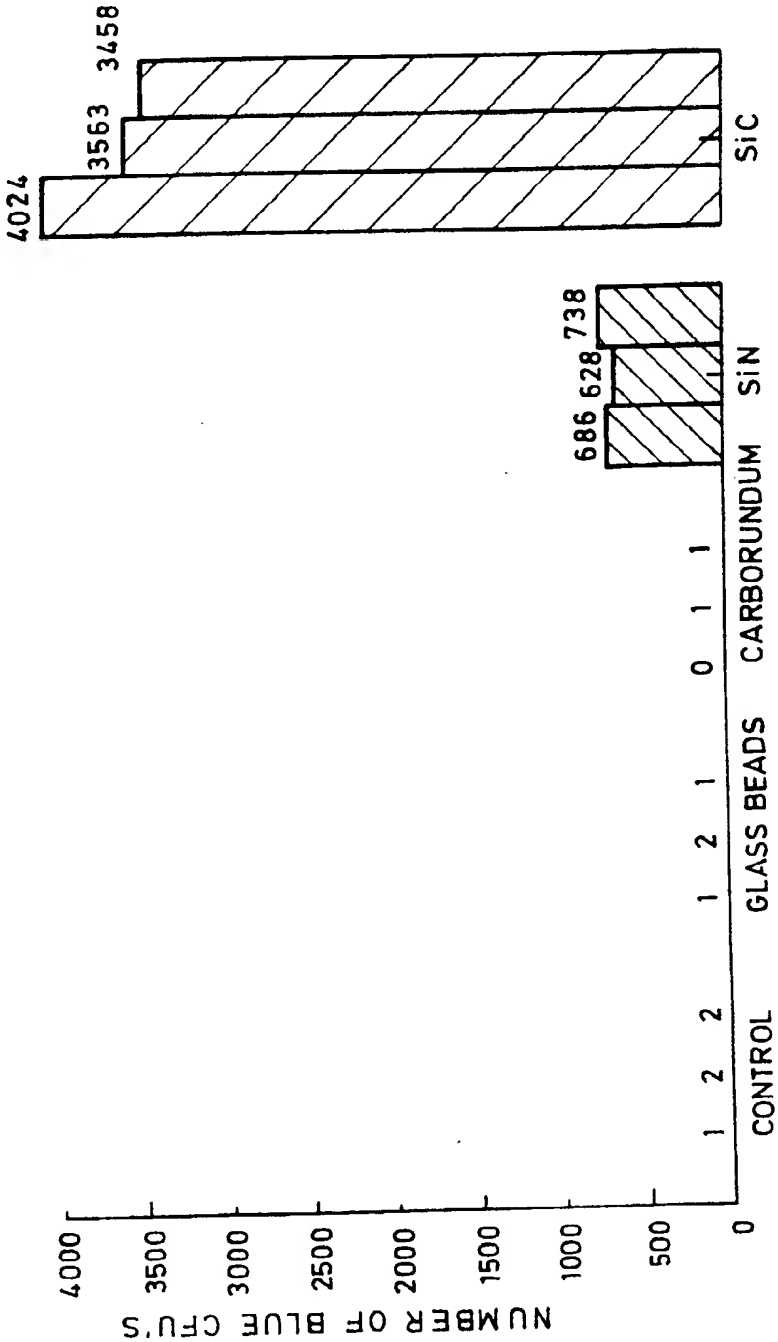
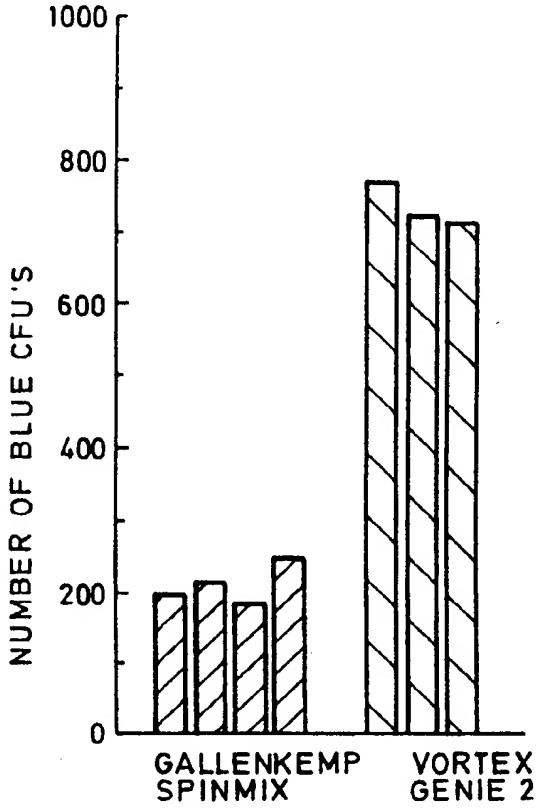


FIG. 3 DNA TRANSFORMATION OF BMS WITH p35S-PAC COMPARING THE GALLENKAMP SPINMIX AND THE VORTEX GENIE 2



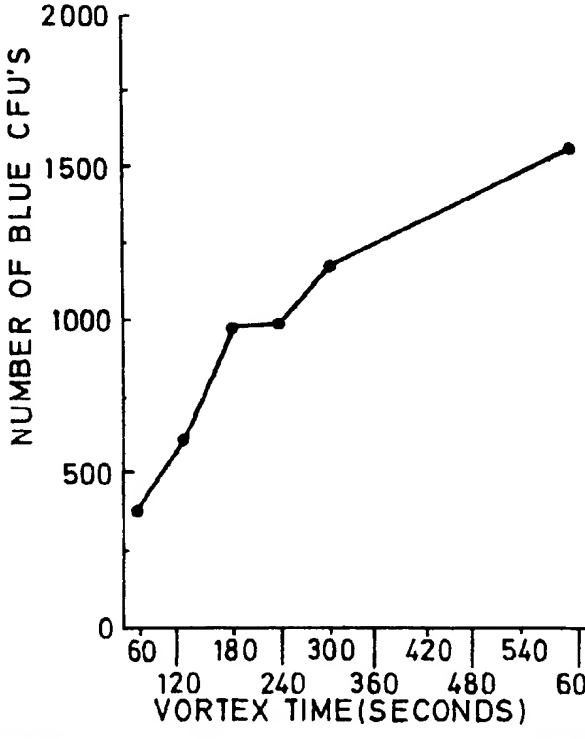


FIG. 4

A TIME COURSE FOR BMS TRANSFORMED WITH p35S-PAC USING SILICON CARBIDE FIBRES AND THE VORTEX GENIE 2

FIG. 5

A TIME COURSE FOR BMS TO DETERMINE THE EFFECTS OF VORTEX TIME ON CELL VIABILITY, USING THE VORTEX GENIE 2

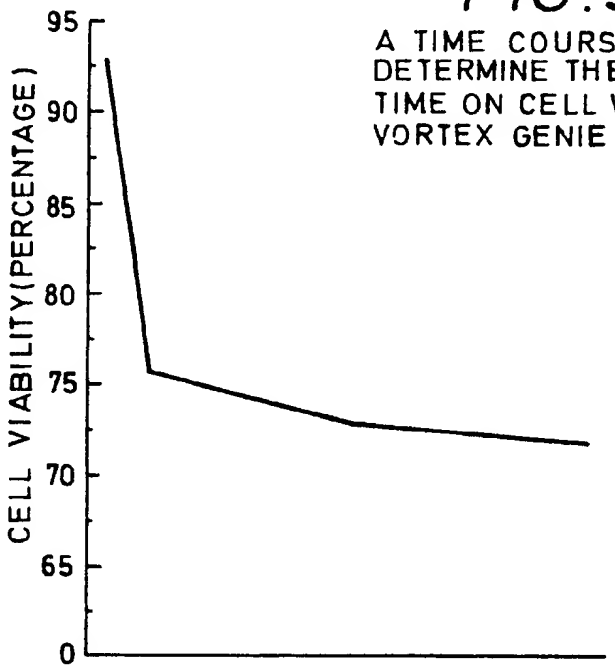


FIG. 6 DNA TRANSFORMATION OF BMS, COMPARING THE GALLENKEMP SPINMIX AND TWO DENTAL AMALGAM MIXERS

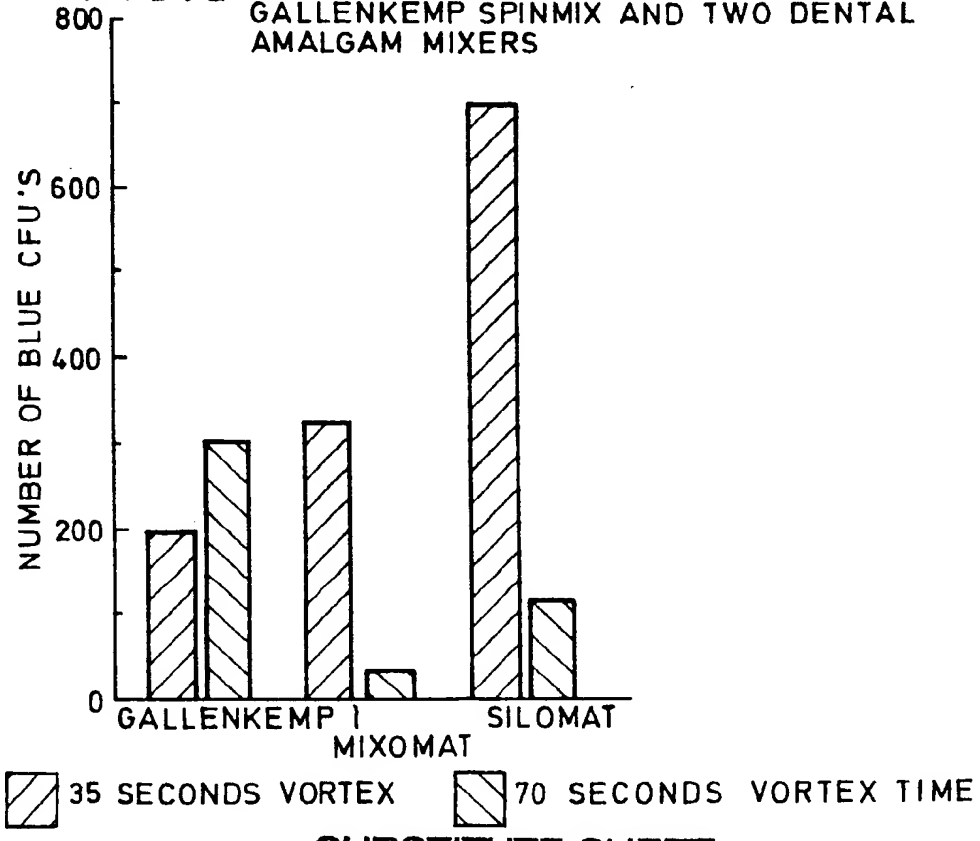


FIG. 7

EFFECTS OF MIXOMAT TREATMENT TIME ON TRANSIENT GUS EXPRESSION IN BMS

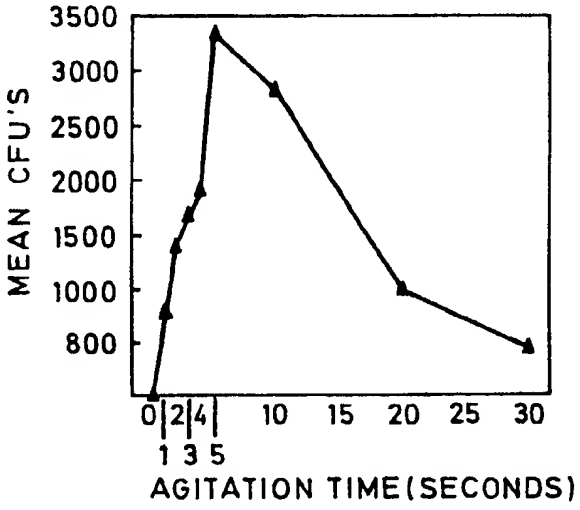


FIG. 8

THE EFFECT OF PRE-MIXING OF WHISKERS WITH DNA ON GUS EXPRESSION IN A5D1-C6

PREMIX DURATION EFFECT ON TRANSIENT EXPRESSION OF pMRP8 IN SiC FIBRE TREATED A5D1 C6

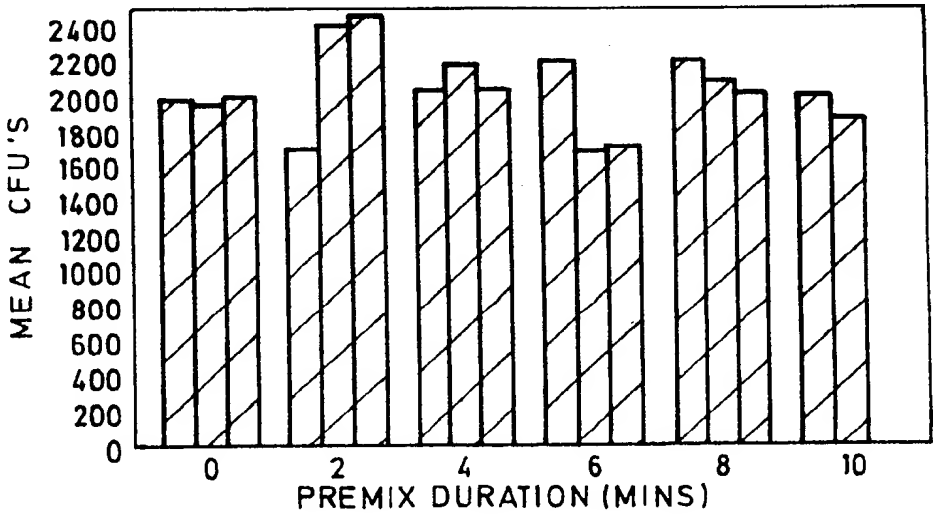


FIG. 9

SOUTHERN BLOT OF FIVE BIALAPHOS RESISTANT GUS EXPRESSING CALLUS LINES

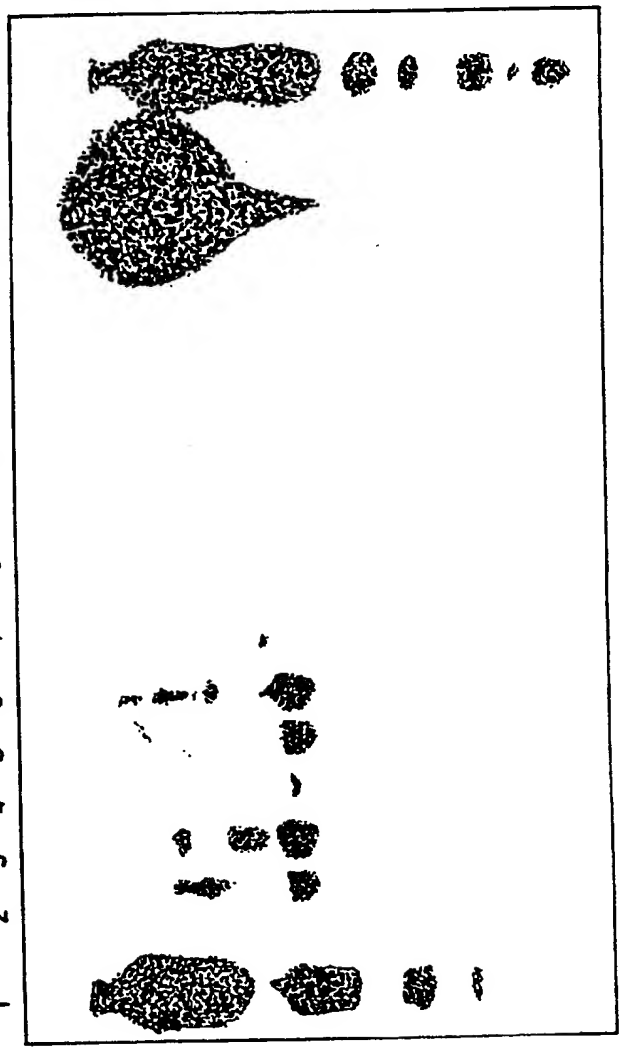
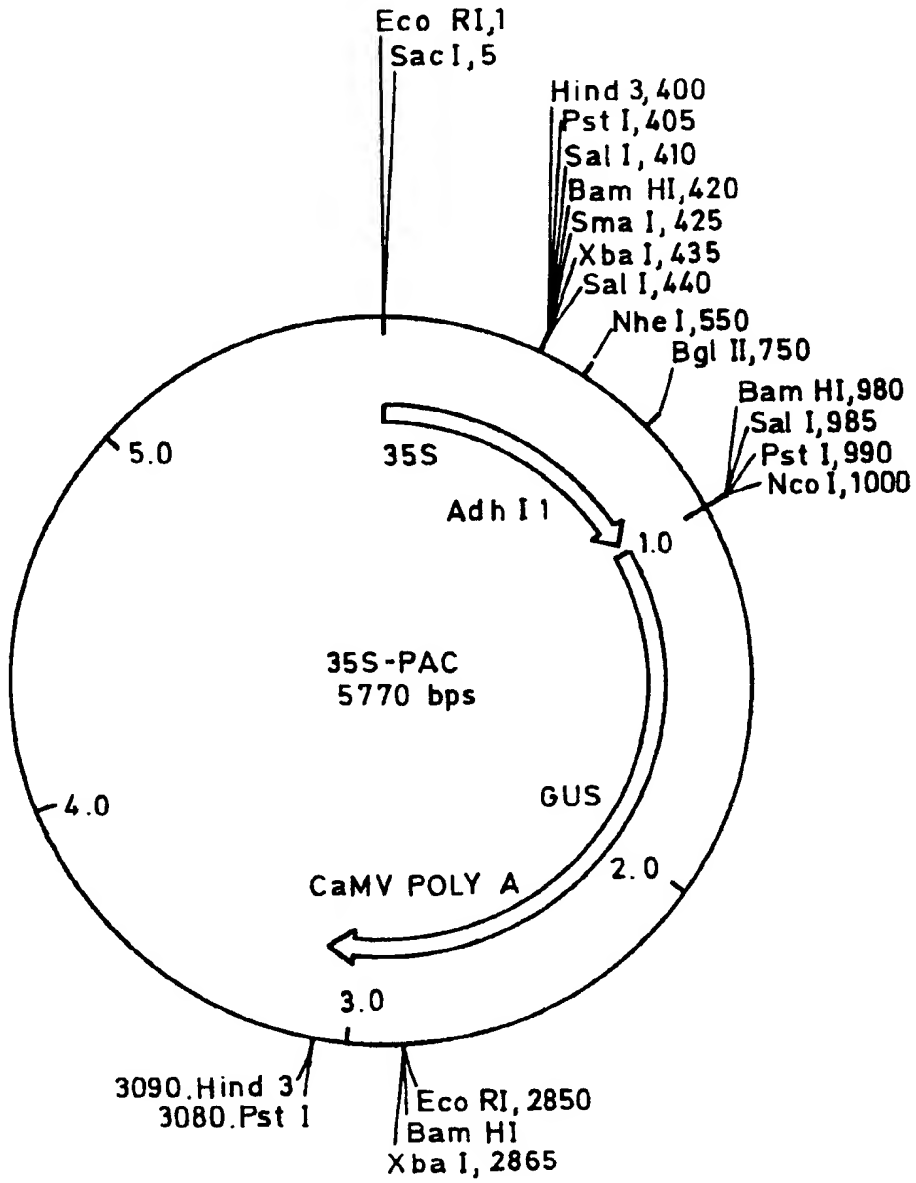


FIG. 10 (1/4)


Eco RI,1
Sac I,5
Hind 3,400
Pst I,405
Sal I, 410
Bam HI,420
Sma I, 425
Xba I,435
Sal I,440
Nhe I,550
Bgl II,750
Bam HI,980
Sal I,985
Pst I,990
Nco I,1000
5.0
35S
Adh I 1
1.0
35S-PAC
5770 bps
GUS
4.0
2.0
CaMV POLY A
3.0
3090.Hind 3
3080.Pst I
Eco RI, 2850
Bam HI
Xba I, 2865

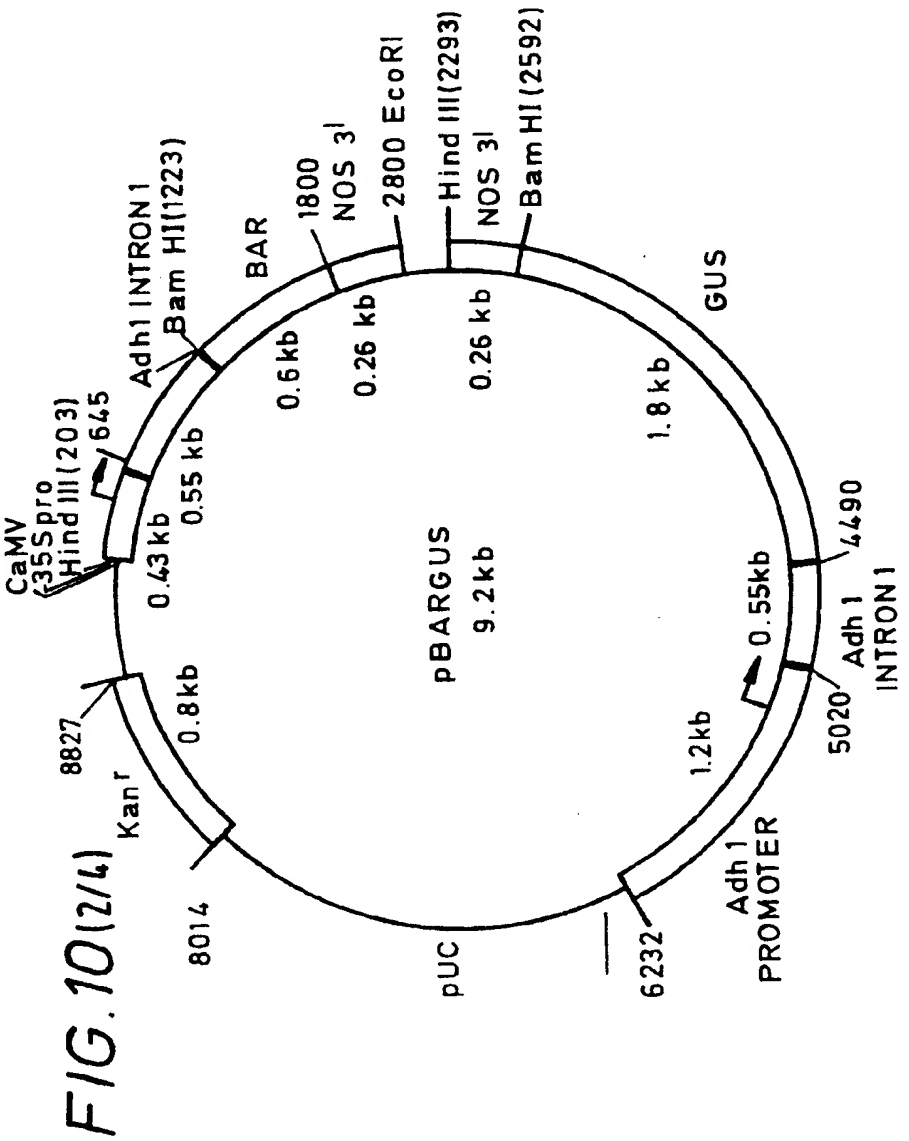

pBARGUS
9.2kb
CaMV (35S pro
Hind III (203)
645
Adh1 INTRON 1
Bam HI (1223)
BAR
1800
NOS 3'
2800 EcoRI
Hind III (2293)
NOS 3'
Bam HI (2592)
GUS
4490
Adh 1
INTRON 1
5020
Adh 1
PROMOTER
6232
pUC
8014
Kan r
8827
0.43 kb
0.55 kb
0.6 kb
0.26 kb
0.26 kb
1.8 kb
0.55kb
1.2kb
0.8kb

FIG. 10 (2/4)

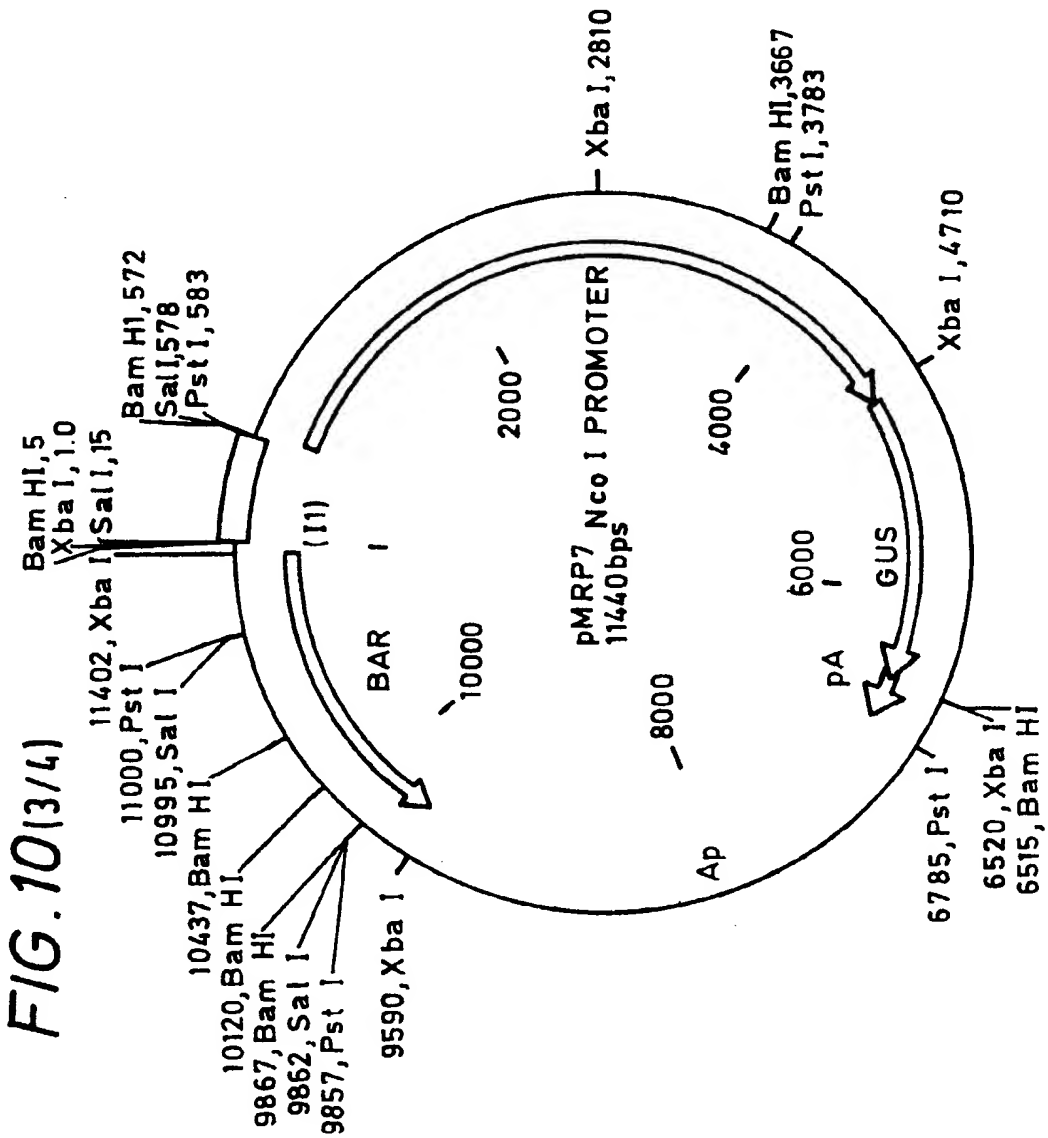
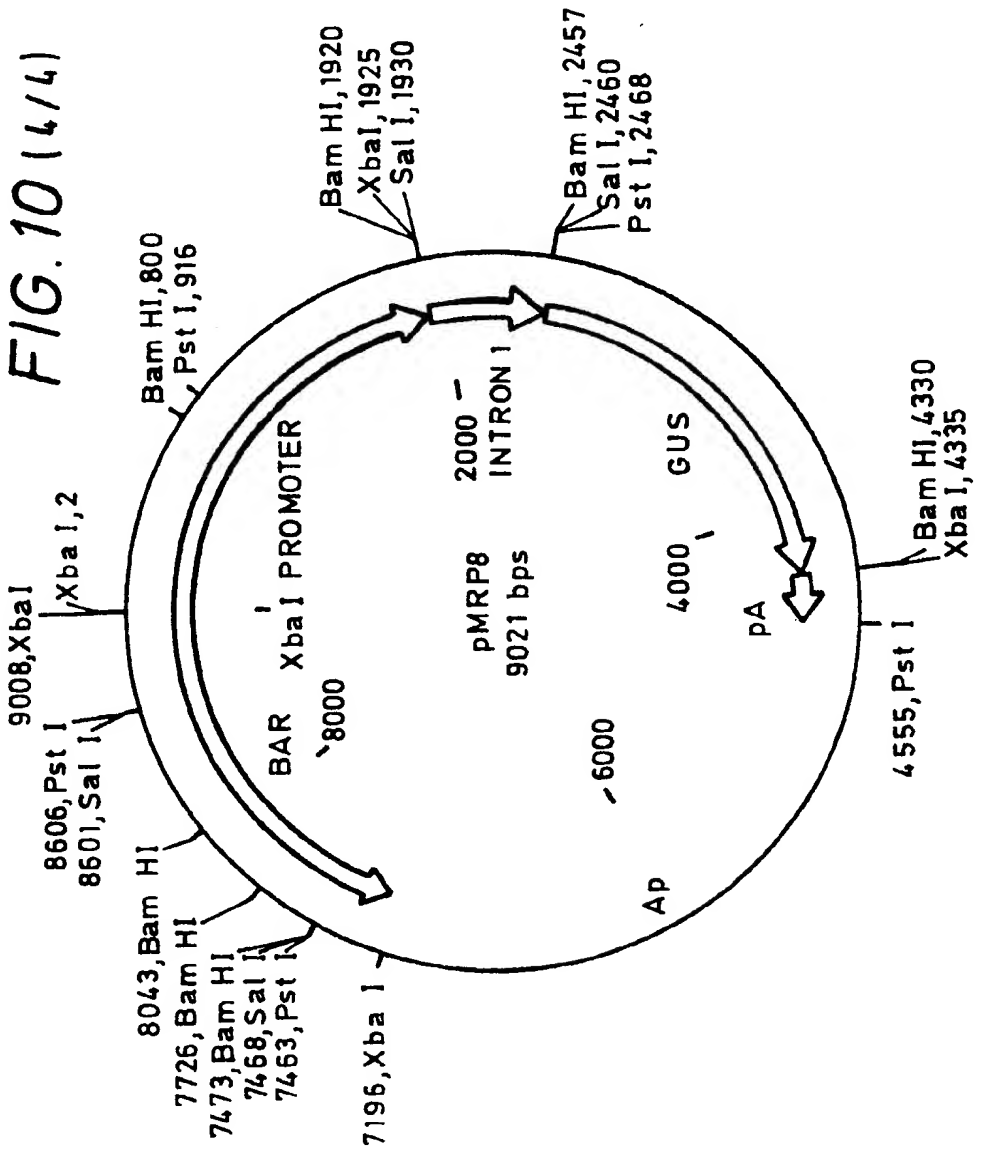

Xba I, 2810
Bam HI, 3667
Pst I, 3783
Xba I, 4710
Bam HI, 572
Sal I, 578
Pst I, 583
Bam HI, 5
Xba I, 1.0
Sal I, 15
11402, Xba I
11402, Pst I
11000, Pst I
10995, Sal I
10437, Bam HI
10120, Bam HI
9867, Bam HI
9862, Sal I
9857, Pst I
9590, Xba I
6785, Pst I
6520, Xba I
6515, Bam HI
pMRP7
11440bps
Nco I PROMOTER
BAR
(11)
GUS
pA
Ap
2000
4000
6000
8000
10000

FIG. 10(3/4)

FIG. 10 (4/4)


Bam HI, 1920
Xba I, 1925
Sal I, 1930
Bam HI, 2457
Sal I, 2460
Pst I, 2468
Bam HI, 800
Pst I, 916
9008, Xba I
Xba I, 2
8606, Pst I
8601, Sal I
8043, Bam HI
7726, Bam HI
7473, Bam HI
7468, Sal I
7463, Pst I
7196, Xba I
BAR
Xba I PROMOTER
8000
2000
INTRON 1
pMRP8
9021 bps
6000
GUS
4000
pA
Ap
Bam HI, 4330
Xba I, 4335
4555, Pst I

INTERNATIONAL SEARCH REPORT

Later... application ...

PCT/GB 93/01162

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/87

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT CELL REPORTS vol. 9 , November 1990 pages 415 - 418 KAEPPLER, H.F., ET AL. 'Silicon carbide fiber-mediated DNA delivery into plant cells' cited in the application see the whole document ---	1-6,10, 11
X	DATABASE WPI Section Ch, Week 9045, Derwent Publications Ltd., London, GB; Class B04, AN 90-341996 & US,A,7 472 538 (AGRIC RES SERVICE) 16 October 1990 see abstract --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 1994	22 -02- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THOER. APPL. GENET. vol. 84 , 1992 pages 560 - 566 KLAEPPER, H.F., ET AL. 'Silicon carbide fiber-mediated stable transformation of plant cells' cited in the application see the whole document	1-6,8,9
A	EP,A,0 331 083 (SCHWEIZERISCHE EIDGENOSSENSCHAFT ETH) 6 September 1989 see claim 19	7
A	THE PLANT CELL. vol. 2, no. 7 , July 1990 , ROCKVILLE, MD, USA. pages 603 - 618 GORDON-KAMM, W.J.,ET AL. 'Transformation of maize cells and regeneration of fertile transgenic plants' see the whole document	9-11
A	THE PLANT CELL. vol. 4, no. 12 , December 1992 , ROCKVILLE, MD, USA. pages 1495 - 1505 D'HALLUIN, K., ET AL. 'Transgenic maize plants by tissue electroporation' see the whole document	12,13
A	CHEMICAL ABSTRACTS, vol. 104, no. 23, 1986, Columbus, Ohio, US; abstract no. 201545, PASTENAK, T.P., ET AL. 'Genetic transformation of higher plant cells by DNA microinjections' see abstract & IZV. AKAD. NAUK SSSR, SER BIOL. no. 2 , 1986 pages 314 - 316	14
A	WO,A,92 12250 (DEKALB) 23 July 1992 see examples 4,10	7,14

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No
PCT/GB 93/01162

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0331083	06-09-89	AU-A-	3090489	07-09-89
		JP-A-	2016986	19-01-90
WO-A-9212250	23-07-92	AU-A-	9176991	17-08-92
		EP-A-	0564595	13-10-93